Characterization of an Allele-Nonspecific Intragenic Suppressor in the Yeast Plasma Membrane H^+-ATPase Gene (*PMA1*)

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ABSTRACT

We have analyzed the ability of *A165V, V169I/D170N*, and *P536L* mutations to suppress *pma1* dominant lethal alleles and found that the *P536L* mutation is able to suppress the dominant lethality of the *pma1-R271T*, *-D378N*, *-D378E*, and *-K474R* mutant alleles. Genetic and biochemical analyses of site-directed mutants at Pro-536 suggest that this amino acid may not be essential for function but is important for biogenesis of the ATPase. Proteins encoded by dominant lethal *pma1* alleles are retained in the endoplasmic reticulum, thus interfering with transport of wild-type Pma1. Immunofluorescence studies of yeast conditionally expressing revertant alleles show that the mutant enzymes are correctly located at the plasma membrane and do not disturb targeting of the wild-type enzyme. We propose that changes in Pro-536 may influence the folding of the protein encoded by a dominant negative allele so that it is no longer recognized and retained as a misfolded protein by the endoplasmic reticulum.

THE yeast plasma membrane H^+-ATPase from *Saccharomyces cerevisiae* is an electrogenic proton pump essential for nutrient uptake and intracellular pH regulation (see review by Serrano 1991). The ATPase belongs to the P-type, ion-translocating ATPase family, which forms an acyl-phosphate intermediate during catalysis and is sensitive to vanadate (Pedersen and Carafoli 1987).

The genes encoding members of the P-type ATPase family from bacterial, fungal, plant, and animal cells have been cloned and sequenced, in each case allowing the primary structure of the protein to be deduced. Comparison of the P-ATPases' amino acid sequences showed that they share several regions of homology and a common topology (Serrano 1989; Goffeau and Green 1990; Wach *et al.* 1992). Site-directed mutagenesis and intragenic suppressor analysis have been used to probe structure-function relationships of the yeast enzyme. Site-directed mutagenesis has allowed the identification of a series of mutations that cause cell growth arrest even when wild-type Pma1 protein is coexpressed (Harris *et al.* 1994; Portillo 1997). Expression of these dominant lethal proteins leads to the accumulation of mutant and wild-type proteins in subplasma membrane structures probably derived from the endoplasmic reticulum (ER; Harris *et al.* 1994). From intragenic suppression analysis of selected *pma1* alleles, three mutations (*A165V*, *V169I/D170N*, and *P536L*) were identified that exhibited characteristics of allele-nonspecific suppressors, as they were able to suppress mutations located either at the carboxyl terminus (Eraso and Portillo 1994) or at the ATP-binding domain of the enzyme (Maldonado and Portillo 1995). Similar allele-nonspecific suppressors have been isolated in other systems and appear to affect protein stability (Shortle and Lin 1985; Thomas *et al.* 1991). In this report, we show that the *P536L* mutation is able to suppress dominant lethal *pma1* mutations located in three different regions of the ATPase. Further analysis of site-directed mutants at Pro-536 suggests that this residue is important for biogenesis of the enzyme and that suppression of dominant lethality is probably caused by compensatory structural alterations that alleviate retention of the mutant protein at the ER.

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MATERIALS AND METHODS

Yeast strains and growth conditions: Yeast strain XZ611 is GAL$^+$ *MAT***a** *ura3 leu2 trp1* (Harris *et al.* 1994), and it was used to determine the dominant lethality of *pma1* genes and in immunofluorescence experiments. Strain SY4 is GAL$^+$ *MAT*a *ura3 leu2 his4 sec6 pma1*::*YIpGAL-PMA1* and was used for expression of the ATPase in secretory vesicles (Nakamoto *et al.* 1991). Synthetic media with 2% dextrose (SD), 2% lactic acid (SL), or 2% galactose (SG) and the appropriate requirements were used (Rose *et al.* 1990). Yeast cells were transformed using the lithium acetate procedure (Ito *et al.* 1983). To test the dominant lethality of the *pma1* alleles, transformants were transferred to SL medium and, after growth, suspended in water at a cell density of 2×10^7 cell/ml, and 5 μl was dropped on SG.

Construction of pma1 alleles: The *pma1* genes containing Pro-536 mutations were produced by site-directed mutagenesis. The Amersham Kit for oligonucleotide-directed mutagenesis (Amersham Corp., Arlington Heights, IL) was used to intro-

duce the mutations into a 2.2-kb *Xba*I fragment of the *PMA1* gene previously subcloned into M13mp19. After mutagenesis, the entire *Xba*I fragment was sequenced to verify that only the nucleotide changes introduced by the mutagenic oligonucleotides were obtained. The *Xba*I fragments containing the mutation were moved into the full-length PMA1 gene in plasmid pRS427 (Vallejo and Serrano 1989). Plasmid pRS427 is a derivative of the *URA3* single-copy plasmid YCp50 (Rose and Broach 1991) and containts the *PMA1* gene joined to the *GAL1* promoter. The *pma1* alleles in Tables 1 and 3 were constructed by placing the *pma1* genes containing dominant lethal mutations under the control of the galactose-inducible promoter in plasmid pRS427, as described in Portillo (1997). The Pro-536 mutations were subsequently combined in *cis* with the different dominant lethal mutations by exchange of a 2.2-kb *Xba*I fragment containing the allele-nonspecific mutations with the corresponding fragment in the dominant lethal *GAL1::pma1* gene construction. All mutant constructions were verified by sequencing. The hemagglutinin (HA)-tagged *pma1* alleles used in this study were generated by exchange of a 3.4-kb *Bst*EII-*Hin*dIII fragment containing the desired mutations with the corresponding fragment in plasmid pFP302. The plasmid pFP302 is identical to pRS427, except that it carries an HA-tagged wild-type *PMA1* under the control of the *GAL1* promoter. The nine–amino acid HA epitope was introduced after the second amino acid of the ATPase by site-directed mutagenesis (Portillo 1997). All mutant constructions were verified by sequencing.

When coexpression of dominant lethal and wild-type proteins was required, cells were transformed with a pRS427 derivative containing different dominant lethal mutations and plasmid pFP239. The plasmid pFP239 is a derivative of the *LEU2* single-copy plasmid pSB32 (Rose and Broach 1991) and contains an HA-tagged wild-type *PMA1* under the control of the *GAL1* promoter.

Replacement of the P536N and P536L mutant alleles by the chromosomal wild-type PMA1: The ATPase gene fragments containing the Pro-536 substitutions were subcloned by exchange of a 2.2-kb *Xba*I fragment in plasmid pFP36. Plasmid pFP36 is a derivative of pUC18 and contains a PMA1 gene in which a *URA3* marker was introduced into a synthetic *Xho*I linker located at the intergenic region between *PMA1* and *LEU1* (Serrano *et al.* 1986). Strain FPY183 was transformed with the *Hin*dIII *pma1::URA3* fragments containing the Pro-536 mutations. Strain FPY183, derived from autodiploid W303 (Portillo 1997), is heterozygous for a *LEU2*-marked deletion of *PMA1* in which the 3.37-kb *Eco*RV-*Sal*I *PMA1*–coding region was replaced with a 2.3-kb *LEU2* fragment. After transformation, Ura^+ Leu^- diploids were selected and tetrads were dissected. The diploid *pma1-P536N::URA3/PMA1* yielded only two Ura^- viable spores, and the *pma1-P536L::URA3/PMA1* rendered four viable spores. In each case, two independent diploids were dissected and segregation of 15 tetrads of each were studied. After growth and analysis of the Ura^+ segregants containing the *P536L* mutation, genomic DNA-containing portions of the *pma1-P536L* gene were amplified by polymerase chain reaction and sequenced to ensure that only the *P536L* mutation was present.

Fluorescence microscopy: Strain XZ611, transformed with different *pma1* alleles, was selected in SD medium. To induce the *GAL1::pma1* gene expression, cells were cultured in SL medium overnight at 30°, collected, resuspended in SG medium, and cultured for 5 hr at 30°. Cells were fixed and stained for immunofluorescence as described (Pringle *et al.* 1991). Localization of HA-tagged Pma1 was determined by immunofluorescence using a rhodamine-conjugated anti-HA antibody (Boehringer Mannheim, Mannheim, Germany).

Expression of the ATPase in secretory vesicles: The 2.6-kb *Bam*HI-*Hin*dIII fragments containing the Pro-536 substitutions were ligated into plasmid pPMA1.2 (Nakamoto *et al.* 1991) that had been digested with *Bam*HI and *Hin*dIII. After the fragments were moved into plasmid pPMA1.2, a 3.77-kb *Sac*I-*Hin*dIII fragment containing the entire ATPase coding sequence was cloned into vector YCp2HSE (Nakamoto *et al.* 1991), placing the mutant alleles under the control of a heat-shock-inducible promoter. The resulting plasmids were transformed into strain SY4 (Nakamoto *et al.* 1991). This strain has the chromosomal *PMA1* gene under the control of the *GAL1* promoter and also carries the sec6-4 mutation that blocks fusion of secretory vesicles with the plasma membrane. For expression studies, transformed SY4 were grown to mid-exponential phase ($OD_{660}\sim0.5$) on SG and shifted to SD for 3 hr to turn off transcription of chromosomal *PMA1* and then shifted to 37° for 2 hr to turn on expression of the plasmid-borne gene and block fusion of secretory vesicles with plasma membrane. Secretory vesicles were then isolated by differential centrifugation as described (Nakamoto *et al.* 1991).

Biochemical methods: Plasma membranes were purified from glucose-metabolizing yeast by differential and sucrose gradient centrifugation (Serrano 1983). Immunoquantification of the ATPase was performed as described (Rao and Slayman 1993). ATPase activity was assayed at pH 6.5 with 5mm ATP (Serrano 1988). H^+ pumping into secretory vesicles was monitored by fluorescence quenching of acridine orange (Nakamoto *et al.* 1991). Proton efflux from the cells was measured after starvation at 4° and glucose addition at 30° (Serrano 1980). Protein concentration was determined by the Bradford method (Bradford 1976) with the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA) and bovine IgG as standard. The monoclonal antibodies Mab12 against the yeast ATPase (Serrano *et al.* 1993) and 12CA5 against the HA epitope (Wilson *et al.* 1984) were used in immunoquantification and immunoblot. Plasma membrane proteins were separated by SDS-PAGE on 8% acrylamide using the Laemmli system (Laemmli 1970). Western blot with second antibody conjugated to alkaline phosphatase (Bio-Rad) was as described (Blake *et al.* 1984).

RESULTS

Suppression spectrum of allele-nonspecific suppressors: The *A165V, V169I/D170N*, and *P536L* mutations were selected previously by suppression of the otherwise unrelated *S911A/T912A* (Eraso and Portillo 1994) and *K474R* (Maldonado and Portillo 1995) mutations. The fact that the *K474R* mutation is a dominant lethal allele (Portillo 1997) prompted us to examine the effect of the *A165V, V169I/D170N*, and *P536L* mutations on the dominant lethality of several other *pma1* mutations located in different regions of the enzyme. For this, we created novel combinations of dominant lethal and suppressor mutations in *cis*, and each of the new *pma1* alleles was placed under the control of the *GAL1* promoter (see materials and methods). Plasmids carrying the *GAL1::pma1* alleles were used to transform a wild-type strain. Under these conditions, yeast cells carrying a dominant lethal allele arrest growth when they are transferred from glucose- to galactose-containing medium. The results of this analysis for dominant lethality of the different *in vitro*-generated alleles

TABLE 1

Suppression of dominant lethal mutations by allele-nonspecific suppressors

Dominant lethal mutations	Allele-nonspecific suppressors		
	A165V	*V169I/D170N*	*P536L*
D200N	nd	nd	−
E233Q	nd	nd	−
R271T	−	−	+
P335A	−	−	−
D378N	−	−	+
D378E	−	−	+
D378T	−	−	−
K474R	+	+	+

Mutations were combined in *cis*, and the resulting alleles were placed under the control of the galactose-inducible promoter. Suppression is defined as the ability of the XZ611 yeast strain to grow on SG medium.

+, suppression; −, no suppression; nd, combination of mutations was not done.

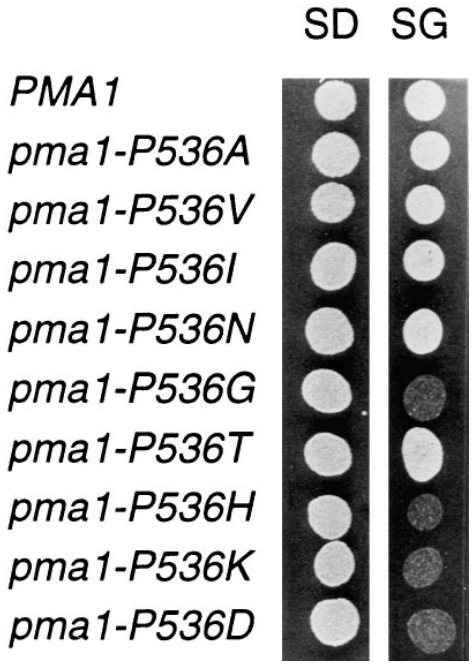

Figure 1.—Spot test for growth of yeast strains expressing dominant lethal alleles of *PMA1*. Strains are recombinant, carrying the *PMA1* wild-type allele or the indicated *pma1* allele under the control of the galactose-inducible promoter. The test was performed as indicated in materials and methods. Plates were photographed after 36 hr at 30°.

are presented in Table 1. Only *P536L* reversion could suppress, in addition to *K474R*, several other dominant lethal mutations tested.

Genetic and biochemical analyses of Pro-536 substitution: To examine the effect of amino acid replacement on suppression ability, we created by site-directed mutagenesis new *pma1* alleles in which Pro-536 was replaced with residues of different size and charge (Pro-536 → A, V, I, N, G, T, H, K, D). Before the above-mentioned analysis, the phenotype of the *pma1* alleles generated was determined. We first examined whether or not the new mutations were dominant lethal. For this, the mutant ATPase genes were placed under control of the *GAL1* promoter and transformed into the appropriate wild-type yeast to test for growth in galactose-containing medium. The results of this test for dominant lethality of the different site-directed mutations are summarized in Figure 1. Substitution of Pro-536 by either small uncharged (Gly) or charge residues (His, Lys, and Asp) renders a dominant lethal allele.

The expression of dominant lethal proteins blocks their transport to the plasma membrane, and the proteins are accumulated into cytoplasmic structures called "Bip bodies" (Supply *et al.* 1993; Harris *et al.* 1994). The expression of dominant negative protein also blocks the transport of wild-type Pma1 and leads to its colocalization in the same structures (Harris *et al.* 1994). To date, all dominant lethal *PMA1* alleles identified exhibit the same phenotype (Harris *et al.* 1994; Portillo 1997). To test whether the dominant lethal mutations detected here also interefered with wild-type Pma1 targeting to the plasma membrane, we studied the transport of wild-type ATPase to the cell surface when dominant negative alleles are also expressed. To this end, we introduced the HA epitope in the wild-type Pma1 protein, and both the HA-tagged and dominant lethal allele were coexpressed under the control of the *GAL1* promoter in the same cell. The location of the wild-type HA-tagged protein was studied in galactose-cultured cells by immunofluorescent staining of wild-type Pma1 with the anti-HA monoclonal antibody (Figure 2). When HA-tagged wild-type protein was induced simultaneously with the dominant lethal protein, the anti-HA antibody decorated a set of cytoplasmic structures similar to those observed for other dominant negative Pma1 proteins (Harris *et al.* 1994; Portillo 1997). When only the HA-tagged dominant negative proteins were expressed under the control of the *GAL1* promoter, the same staining of cytoplasmic dots was observed (data not shown). These results suggest that dominant lethality of *pma1-P536G*, *-P536H*, *-P536K*, and *-P536D* is caused by the interference of the mutant protein with the transport of wild-type Pma1 protein.

The secretion of the nondominant lethal P536A, P536V, P536L, P536I, P536N, and P536T proteins was also studied. We introduced the HA epitope in the mutant proteins, and HA-tagged proteins were expressed under the control of the *GAL1* promoter. We examined the location of each of these HA-tagged mutant proteins by immunofluorescent staining after galactose induction. The anti-HA antibody decorated the cell periphery in all the cases (data not shown), except in cells expressing the HA-tagged P536N protein, in which it accumulated in subplasma membrane structures reminiscent of

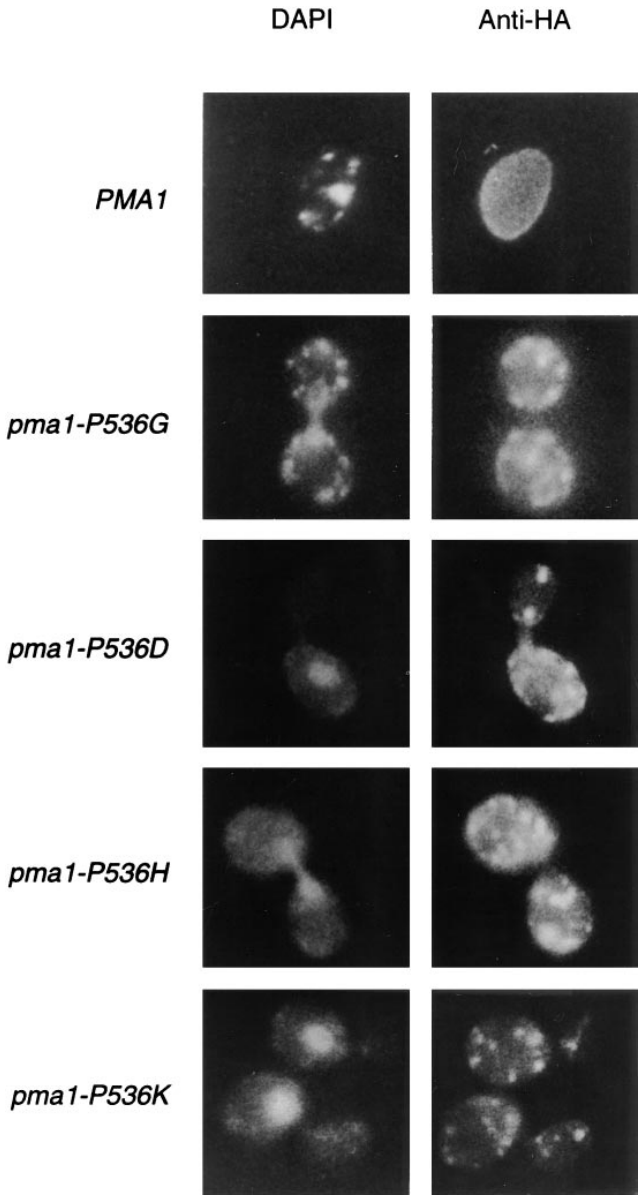

Figure 2.—Accumulation of wild-type *HA-tagged Pma1* in cells expressing dominant lethal *Pro-536* mutations. Transformants carried the HA-tagged *PMA1* gene and either the wild-type or the indicated dominant lethal allele under the control of the *GAL1* promoter. Expression of the *GAL::pma1* genes was induced with galactose for 5 hr. Panels show staining of the nucleus (DAPI) or immunofluorescence using a rhodamine-conjugated anti-HA antibody (anti-HA Ab).

those observed for dominant negative proteins (Figure 3). In contrast to dominant lethal proteins, the P536N protein does not block transport of wild-type Pma1 protein to the cell surface (data not shown). This may indicate that the *P536N* mutation renders a recessive lethal *pma1* allele, which was confirmed by analyzing the segregation of the heterozygous diploid *pma1-P536N::URA3/PMA1* (see materials and methods). After sporulation and tetrad dissection of two independent diploids, only two viable spores/tetrad were ob-

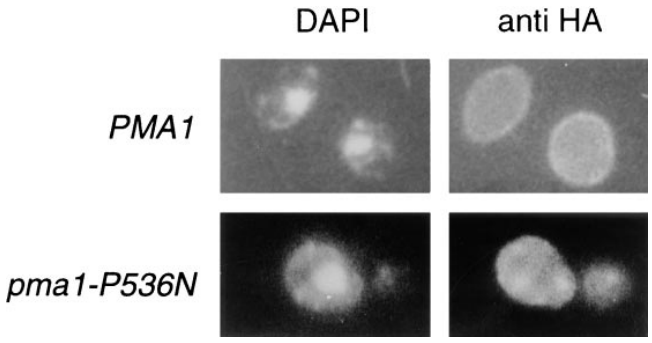

Figure 3.—Accumulation of the recessive lethal HA-tagged P536N protein in cytoplasmic structures. The transformant carried either the HA-tagged *PMA1* or HA-tagged *P536N* genes under the control of the *GAL1* promoter. After galactose induction, cells were stained for the nucleus (DAPI) or for the HA-tagged protein (anti-HA).

tained; both were Ura$^-$ (15 tetrads studied in each of the diploids), thus confirming that *pma1-P536N* is a recessive lethal allele.

Pro-536 is located within the sequence motif ^{534}DPPR, which is fully conserved among all eukaryotic, cation-transporting ATPases (Serrano 1989; Goffeau and Green 1990; Wach *et al.* 1992) and is believed to form part of the ATP-binding site (Rao *et al.* 1989; Serrano 1991). This prompted us to further characterize the effect of Pro-536 substitution on enzyme function. The *P536A*, *P536V*, *P536L*, *P536I*, or *P536T* mutant genes were placed under the control of a heat-shock-inducible promoter in the plasmid YCp2HSE (Nakamoto *et al.* 1991). Plasmids YCp2HSE containing the mutant genes were transformed into strain SY4 (Nakamoto *et al.* 1991). This strain has the chromosomal *PMA1* gene under control of the *GAL1* promoter and also carries the temperature-sensitive *sec6-4* mutation that blocks fusion of secretory vesicles with the plasma membrane. Thus, when cells were incubated in galactose medium at 23°, the chromosomal wild-type ATPase was produced, and when the cells were transferred to glucose medium at 37°, the expression of the wild-type ATPase was repressed and the mutant ATPase was expressed. Because strain SY4 carries a temperature-sensitive *sec6-4* mutation, the shift to 37° also led to the accumulation of the newly synthesized mutant ATPase in secretory vesicles. Secretory vesicles were purified by differential centrifugation for analysis of the newly synthesized mutant ATPase (Nakamoto *et al.* 1991). Table 2 summarizes the behavior of the mutant enzymes. In all the mutants, expression levels were significantly higher than that of the wild-type protein and, similarly, all had relatively high ATPase activity and H^+-pumping rates. After correction for the amount of Pma1, values ranged from 60% hydrolysis/53% pumping in P536A to 114% hydrolysis/93% pumping in P536I. Other kinetic properties assayed, K_m for ATP and K_i for orthovanadate, appeared to be essentially wild type. Thus, it appears that Pro-536

TABLE 2

Effect of Pro536 replacements on expression, ATP hydrolysis, proton transports, and kinetic properties of ATPase

		ATPase[b]				H^+ transport[d]	
Mutation	Amount of ATPase[a](%)	Uncorrected (μmol/min/mg)	Corrected (%)	K_m (ATP)[c] (mm)	K_i (vanadate)[c] (μm)	Uncorrected (%Q/mg)	Corrected (%)
Wild-type	100	0.35	100	1.1	1.0	255	100
None[e]	5	0.02					
P536A	200	0.42	60	0.9	1.3	270	53
P536V	250	0.96	109	1.3	1.0	590	93
P536L	350	1.10	90	1.3	1.0	710	80
P536I	150	0.60	114	1.2	1.5	355	93
P536T	350	1.20	98	1.1	0.9	760	87

[a] Quantified by immunoassay. The value for the wild type was considered 100%.
[b] ATPase activity was assayed at pH 6.5 with 5 mm ATP. Values are the average of two independent experiments. Each mutant value was corrected for expression relative to the wild-type control run in parallel.
[c] The K_m was determined by varying ATP from 0.8 to 6 mm. The K_i was determined by varying the concentration of vanadate from 0 to 50 μm.
[d] Fluorescence quenching of acridine orange was used to monitor pumping of protons into secretory vesicles. Data for mutants were corrected for expression relative to the parallel wild-type control.
[e] Secretory vesicles were isolated from SY4 transformed with the vector YCp2HSE carrying no *PMA1* gene.

is not indispensable for enzyme activity and that the main defect of these Pro-536 substitutions seems to be hyperaccumulation of enzymes in the secretory vesicles.

To determine whether or not the Pro-536 mutations also led to hyperaccumulation of the mutant Pma1 protein at the plasma membrane, we analyzed the transport of wild-type and mutant enzymes to the cell surface. Cells expressing HA-tagged wild-type or mutant proteins under the control of the *GAL1* promoter were shifted from glucose- to galactose-containing medium, and plasma membranes were purified at different induction times and analyzed by Western blot using anti-HA antibody. An example of this analysis is shown in Figure 4 for wild-type and P536L Pma1 protein. The newly synthesized HA-tagged P536L Pma1 protein is accumulated at the plasma membrane at levels higher than those of the wild type. Considering that the wild-type

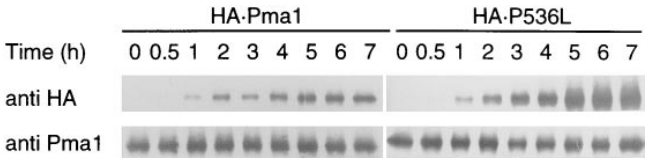

Figure 4.—Accumulation of wild-type and P536L proteins at the plasma membrane. Strains are recombinants carrying either the HA-tagged *PMA1* (HA · Pma1) or HA-tagged *P536L* (HA · P536L) gene. Cells were grown in SL medium and transferred to SG to induce the expression of the *GAL::pma1* genes. At the times indicated, samples were taken and the plasma membrane was purified. Purified plasma membrane was analyzed by immunoblot using monoclonal antibody 12CA5 against the HA epitope (anti-HA) and Mab12 against Pma1 (anti-Pma1). The upper blot was intentionally developed longer than the lower one to detect expressed HA-tagged Pma1 at shorter times.

ATPase is a long-lived and metabolically stable protein with a half-life of >20 hr (Benito *et al.* 1991), the observed hyperaccumulation of newly synthesized P536L protein at shorter times cannot be explained by changes in the stability of the mutant protein. Thus, it seems that Pro-536 may play an important role in the biogenesis of the plasma membrane ATPase.

Suppression spectrum of Pro-536 substitution: After phenotypic characterization of the mutant carrying the Pro-536 mutation, the *pma1-P536A*, *-P536V*, *-P536I*, and *-P536T* alleles were analyzed for competence to suppress the *R271T*, *D378N*, *D378E*, and *K474R* dominant lethal mutations. We combined the Pro-536 substitutions with the dominant negative mutations in *cis* (see materials and methods). The new *pma1* alleles were placed under control of the *GAL1* promoter and tested for dominant lethality. The result of this intragenic suppression analysis is shown in Table 3. The dominant lethality of the *R271T* and *D378N* mutations was suppressed by any of the Pro-536 substitutions tested, and none of the Pro-536 mutations suppressed the lethality of the *pma1-*

TABLE 3

Intragenic suppression of lethal dominant mutations by substitution at Pro-536

	Dominant lethal mutations			
pma1 alleles	*R271T*	*D378N*	*D378E*	*K474R*
P536A	+	+	−	−
P536V	+	+	−	+
P536I	+	+	−	−
P536T	+	+	−	+

Mutations were combined and tested as described in Table 1.

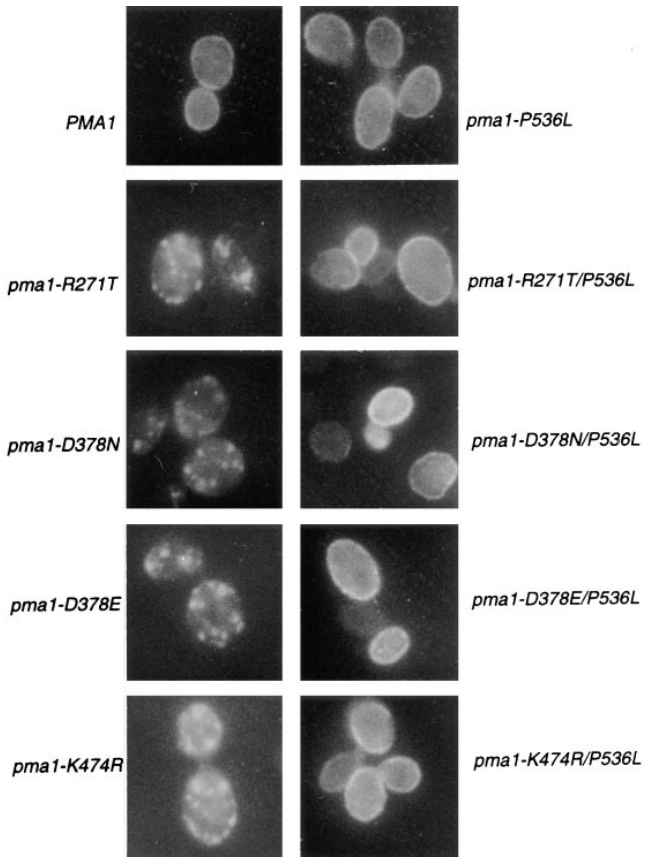

Figure 5.—Effect of *P536L* mutation on localization of dominant lethal Pma1 proteins. Transformants carried the indicated HA-tagged *pma1* allele under the control of the *GAL1* promoter. After galactose induction of the *GAL1::pma1* genes, cells were stained for immunofluorescence with a rhodamine-conjugated anti-HA antibody.

D378E allele. *K474R* was suppressed by the *P536V* and *P536T*, but not by the *P536A* mutations, which suggests that the bulkiness of the amino acid side chain is important for this intragenic suppression.

Effect of Pro-536 substitutions on localization of dominant lethal Pma1: To understand how Pro-536 mutations suppress the dominant lethal alleles, it is necessary to determine whether or not revertant mutant enzymes are still accumulated at Bip bodies. We examined the transport of HA-tagged revertant enzymes. Cells expressing HA-tagged wild-type, dominant lethal, or revertant proteins under the control of the *GAL1* promoter were shifted from glucose- to galactose-containing medium, and the location of the HA-tagged proteins was examined by immunofluorescent staining after galactose induction. Figure 5 shows, as an example, the results obtained with the revertants generated from the *P536L* mutation: *R271T/P536L*, *D378N/P536L*, *D378E/P536L*, and *K474R/P536L*. In contrast to the R271T, D378N, D378T, and K474R dominant negative proteins, the revertant Pma1 proteins are localized at the plasma membrane. This indicates that *P536L* abolishes retention of the proteins encoded by the dominant lethal mutations at the ER and leads to transport of the revertant enzymes to the cell surface.

Phenotype of yeast expressing the *pma1-P536L* allele: The amount of Pma1 in the plasma membrane appears to be tightly regulated, as overexpression of the ATPase gene on a multicopy plasmid in yeast yields only a slight increase in the amount of Pma1 in the plasma membrane (Eraso *et al.* 1987). The fact that P536L mutant enzyme expression significantly increased the amount of mutant Pma1 at the plasma membrane led us to explore the phenotype of a yeast strain expressing only the *pma1-P536L* allele. Two heterozygous diploids, *pma1-P536L::URA3/PMA1* (see materials and methods), were sporulated and tetrads were dissected. In all tetrads studied, four viable spores/tetrad were obtained. Three Ura^+ haploid segregants containing the *pma1-P536L* allele were selected to evaluate the physiological consequences of the P536L mutant protein expression. The results of this study are summarized in Table 4. Yeast expressing the *pma1-P536L* allele showed a twofold increase in the amount of Pma1 compared with the wild-type strain. As a consequence, the *in vitro* ATPase activity assayed in purified plasma membrane was also higher than that of the wild-type strain. Yeast cells actively extrude protons into the medium, an activity caused by plasma membrane H^+-ATPase, which reflects the *in vivo* activity of the enzyme (Serrano 1980; Cid *et al.* 1987) and correlates with both the *in vitro* ATPase activity and the specific growth rate (Portillo and Serrano 1989; Vallejo and Serrano 1989). It was therefore expected that cells exhibiting high *in vitro* ATPase activity would also show high H^+-pumping activity relative to the wild-type cells. The fact that cells expressing the P536L mutant enzyme exhibited lower H^+-pumping activity relative to the wild-type strain suggests that not all of the P536L mutant enzyme accumulated in the plasma membrane is physiologically active or that the mutant enzyme is only partially active. This is reinforced by the fact that the specific growth rate of the mutant cell decreased relative to that of the wild type.

DISCUSSION

Role of Pro-536 in ATPase function: Pro-536 is fully conserved among all known members of the P-type ATPase family (Serrano 1989; Goffeau and Green 1990; Wach *et al.* 1992). The *P536A*, *P536V*, *P536L*, *P536I*, and *P536T* mutations resulted in active enzymes that exhibited kinetic parameters similar to those of the wild-type enzyme; therefore, at least in these cases, Pro-536 does not appear to be essential for enzyme function. The most remarkable phenotype of these enzymes appears to be a hyperaccumulation at the cell surface. In contrast, enzymes bearing *P536G*, *P536H*, *P536K*, and *P536D* mutations were defective in targeting to the plasma membrane, as they are retained at the ER. These

TABLE 4

Growth phenotype, ATPase activity, and expression of P536L mutant ATPase

Allele	Specific growth rate[a,e]	ATPase activity[b,e]	Amount of ATPase[c,e]	H^+-pumping activity[d,e]
PMA1	0.43 ± 0.07	0.90 ± 0.15	1.0	11 ± 0.9
pma1-P536L	0.30 ± 0.05	1.6 ± 0.20	2.0	8 ± 0.5

[a] h^{-1}.
[b] ATPase activity ($\mu mol^{-1} \cdot min^{-1} \cdot$ mg protein^{-1}) was measured in purified plasma membranes with 2 mm ATP at pH 6.5.
[c] Quantified by immunoassay in purified plasma membrane. The value for the wild type was taken as 100%.
[d] Measured in whole cells (nmol $H^+ \cdot min^{-1} \cdot$ mg fresh weight cells^{-1}).
[e] Values are the average of three independent experiments.

results suggest that the residue at position 536 may play an important role in enzyme biogenesis. In wild-type yeast cells, newly synthesized Pma1 is delivered to the plasma membrane via the secretory pathway (Holcomb *et al.* 1988; Chang and Slayman 1991). Several lines of evidence suggest that transport of the protein to the plasma membrane is under a tight control that mediates the retention of either overexpressed wild-type (Supply *et al.* 1993) or aberrant (Harris *et al.* 1994; Portillo 1997) Pma1 proteins at the ER. Although little is known about both the mechanisms that mediate the retention at the ER and the molecular determinants involved in the targeting to the plasma membrane of the ATPase, the finding that P536A, P536V, P536L, P536I, and P536T mutant proteins are hyperaccumulated at the cell surface raises the possibility that these mutants bypass some of the tight control on transport of the protein, thus improving the transport of the mutant enyzme to the cell surface.

We found that yeast cells expressing only the *pma1-P536L* mutant allele exhibited a significantly decreased *in vivo* proton transport rate relative to both activity and abundance of ATPase in purified plasma membrane. This may indicate that not all of the accumulated mutant ATPase is physiologically active. Several hypotheses can be considered to explain this striking phenotype. One hypothesis could be that expression of P536L protein was detrimental to yeast growth. If expression of P536L enzyme were toxic, there would be strong selective pressure during growth for the accumulation of second-site mutations able to suppress the *P536L* mutation. This problem would not affect the P536L protein expressed in secretory vesicles, as the mutant protein is transiently expressed in this case. Nevertheless, we rescued and sequenced the *pma1* allele after yeast growth, and only the P536L mutation was present. However, extragenic mutations cannot be completely discarded. The possibility that P536L mutant protein expression led to saturation of the secretory pathway, resulting in proliferation of internal membranes as a mechanism to control the amount of Pma1 at the plasma membrane (Supply *et al.* 1993; de Kerchove d'Exaerde *et al.* 1996), is discarded based on the following arguments: P536L protein was accumulated during yeast growth in a membrane fraction that copurified with the plasma membrane, and immunofluorescence staining of yeast cells expressing the HA-tagged P536L mutant protein with the anti-HA antibody indicated that the mutant protein was localized exclusively at the cell surface. An alternative hypothesis, which could account for cell surface localization of the mutant enzyme and yet low *in vivo* proton transport, could be that the P536L mutant protein remains trapped in subplasma membrane structures derived from the plasma membrane. Based on the finding that the *MOP2/END4* gene, which is required for receptor-mediated and fluid-phase endocytosis (Raths *et al.* 1993), is also important to control the abundance of Pma1 at the plasma membrane (Na *et al.* 1995), it seems reasonable to think that such subplasma membrane structures could be derived from the plasma membrane by endocytosis. This hypothesis raises the possibility that yeast cells could also control the amount of ATPase at the plasma membrane by regulating its endocytic recycling. Nevertheless, the present results do not allow us to dismiss an alternative model in which hyperaccumulation of ATPase at the plasma membrane triggers a posttranscriptional mechanism that down-regulates the ATPase. This type of down-regulation of ATPase has already been observed and is mediated by the plasma membrane–associated protein kinase YCK1 (Estrada *et al.* 1996). Data allowing discrimination between the various possibilities might be provided by analyzing the expression and activity of P536L protein in mutants altered in endocytosis or YCK1.

Mechanism of suppression of dominant lethal mutations by the P536L mutation: We report here that *P536L* mutation suppresses the dominant lethality of the *pma1-R271T*, *-D378N*, *-D378E*, and *-K474R* mutant alleles. These dominant lethal *pma1* alleles are located in different regions of the ATPase: Arg-271 in the stalk region, Asp-378 in the phosphorylation domain, and Arg-474 in the ATP-binding domain (Rao *et al.* 1989; Serrano 1991). Intragenic suppression analysis of selected *pma1* mutations has suggested a conformational coupling be-

tween the stalk region and both the ATP-binding and -phosphorylation domains (Na *et al.* 1993; Harris *et al.* 1994; Maldonado and Portillo 1995). This structural coupling could explain the suppression pattern of the *P536L* mutation. Some specificity is observed for suppression of the Asp-378 substitution, as the *P536L* mutation suppresses the dominant lethality caused by conservative changes (D378 → N, E), but not that induced by a nonconservative substitution (D378 → T) (Table 1). Some steric requirements are also observed for suppression of the *K474R* and *D378E* mutations. In this case, the bulkiness of the amino acid side chain at position 536 appears to be important for suppression (Table 3). Although dominant lethal proteins are accumulated into cytoplasmic structures (Harris *et al.* 1994), we showed that the *P536L* mutation suppresses the mislocalization defect caused by the dominant lethal mutations, leading to transport of the revertant enzymes to the cell surface. There are several mechanisms by which the *P536L* mutation might correct the mislocalization of R271T, D378N, D378E, and K474R mutant proteins. The easiest interpretation is that revertant proteins are localized more efficiently at the plasma membrane, merely because of the improved transport caused by the *P536L* mutation. This explanation, however, although feasible, does not account for the observed specificity of the intragenic suppressor mutation because not every dominant lethal mutation studied in this work is suppressed by Pro-536 mutations, pointing to a steric requirement for suppression. Suppressor mutations able to correct the mislocalization of mutated proteins have been also found in the human cystic fibrosis transmembrane conductance regulator (CFTR; Teem *et al.* 1993). CFTR functions as a chloride ion channel (Anderson *et al.* 1991; Bear *et al.* 1992) located at the apical membrane of epithelial cells (Denning *et al.* 1992a). In CFTR, the deletion of a single amino acid, phenylalanine, at position 508 (ΔF508), alters the conformation of CFTR (Thomas *et al.* 1992; Thomas and Pedersen 1993) and produces two defects: it disrupts the normal processing of the protein in such a way that the ΔF508 mutant protein is retained in the ER (Cheng *et al.* 1990; Denning *et al.* 1992b; Lukacs *et al.* 1994), and it alters chloride transport (Dalemans *et al.* 1991). Teem *et al.* (1993) isolated intragenic revertants of ΔF508 that partially suppressed both the mislocalization and transport defects caused by the deletion of F508, and they interpreted this finding to mean that suppressor mutations act as compensatory mutations that allow proper folding and normal function of mutant CFTR. Although we have no direct evidence that dominant lethal mutations cause misfolding and misfunction of ATPase, both the pattern and specificity of the suppression observed for *P536L* mutation, together with the fact that suppressor mutations correct the mislocation caused by the dominant lethal mutations, suggest that a mechanism similar to that proposed for intragenic suppressors of ΔF508 could be responsible for suppression of *pma1-R271T*, *-D378N*, *-D378E*, and *-K474R* dominant lethal alleles. Our genetic data cannot define the position of these residues within the tertiary structure of the ATPase, but they indicate that these residues could interact with Pro-536. Such interaction might contribute to the correct folding of the ATPase that is needed for proper localization of the enzyme. If dominant lethal mutations disturb such interactions, Pro-536 suppressor mutations could act as compensatory mutations by restoring those interactions, thus allowing proper folding and transport to the plasma membrane. Conformational analysis of polypeptides containing the dominant lethal and/or the Pro-536 suppressor mutations could provide data supporting this hypothesis.

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